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Extra-Specific Manifestation of Nanoheater’s Position Effect on Distinctive Cellular Photothermal Responses

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ABSTRACT: Subcellular localization of nanoparticles plays critical roles in precision medicine that can facilitate an in-depth understanding of disease etiology and achieve accurate theranostic regulation via responding to the aiding stimuli. The photothermal effect is one extensively employed strategy that converts light into heat stimulation to induce localized disease ablation. Despite diverse manipulations that have been investigated in photothermal nanotheranostics, influences of nanoheaters’ subcellular distribution and their molecular mechanism on cellular heat response remain elusive. Herein, we disclose the biological basis of distinguishable thermal effects at subcellular resolution by localizing photothermal upconversion nanoparticles into specific locations of cell compartments. Upon 808 nm light excitation, the lysosomal cellular uptake initialized by poly-ethyleneamine (PEI) modified nanoheaters promoted the mitochondria apoptosis through the activation of Bid protein, whereas the cell surface nanoheaters anchoring via metabolic glycol-biosynthesis triggered the necrosis by direct perturbation of membrane structure. Intriguingly, these two different thermolyses revealed similar levels of heat shock protein expression in live cells. This study stipulates insights underlying the different subcellular positions of nanoparticles for the selective thermal response, which provides valuable perspectives on optimal precision nanomedicine.

KEYWORDS: upconversion nanoparticles, photothermal, NIR light, lanthanide, heat response

Within the three-dimensional cell structure, subcellular compartments possess a substantial implication in locally activating signal transduction, trafficking essential components and conveying bio-molecules to manipulate cell events as well as downstream stress effectors.1,2 Comprehension on how to specifically monitor organelles functions, and to spatiotemporally regulate the unique signalling pathways in a sophisticated cell environment offers great possibility for in-depth understanding of the etiology of disease process. These conceptions can be fully employed for the facilitation of targeted theranostics with maximum efficacy.3,9 Among the various endeavours for such high accuracy, the strategy based on light irradiations, especially utilizing the near-infrared (NIR) spectral window, represents an extraordinary option to achieve deep-tissue penetration and minimize side effects of ever-present biomolecules and endogenous chromophores.10-13

As one typical light-responsive therapeutic modality, photothermal treatment (PTT) directly utilizes the photothermal effects in nanostructures that are capable of absorbing and converting photon energy to heat. Such light-activated thermal effect can ablate targeted diseases cells, thus engendering their promising applications in precision nanomedicine.14-18 Thus far, a plethora of photothermal nano-absorbers have been developed with a main focus on the strategies to improve biocompatibility, specific diseases affinity and photothermal sensitivity for maximum thermal regulation.19-23 However, the biological basis conferring the cellular response to PTT is still under controversy. In spite of the possibility of heat response through the expression of heat shock proteins (HSPs) as an intrinsic cytotoxic effect, their cochaperone interactions and molecular mechanism remain elusive.24 Moreover, the cell death pathways including apoptosis or uncontrolled cell dying, namely necrosis, occurred in photothermal response to severe heat stress have also been intensively investigated but are mostly dependent on the temperature controls by the different light treatment settings including irradiation duration, laser types and the power intensity.25,26

Considering the importance of subcellular compartments in cell function mediations, organelle specific localization of thermal-induced nanomaterials is critical for PTT precision with ultimate efficacy. Furthermore, attributed to the biological diversity in molecule components, native physiological properties and their relative proximity to heat-sensors, subcellular compartments can be affected divergently by localized thermal stress which might cause varied cellular responses.27-29 However, despite the abundant designs of photothermal nano-heaters with the significant efficiency, studies on their subcellular distribution and distinguishable effects on photothermalysis are still unconceivably scarce.

Herein, we selected the rare-earth upconversion nanoparticles with surface coated polydopamine (termed as UPDAs) as a representative model of tissue penetrable NIR light-activated nano-heaters for dissecting intracellular behaviours to heat shock responses, mostly due to their unique photoluminescence properties.30-35 Different intracellular recognition of UPDAs can be achieved by selective moderation of particles surface with poly-ethyleneimine (PEI) and membrane-associated bioorthogonal moieties. Typically, UPDAs were coated with PEI to provide enhanced cell uptake for efficient cytoplasm localization. We incorporated a monosaccharide precursor, peracetylated N-azidoacetylmannosamine (Ac,ManNAz) modified with a bioorthogonal azido tag, N3, on cell surface through the process of intrinsic glycan metabolism. Meanwhile, nanoparticles functionalized with DBCO (dibenzyl cyclooctyne) were conjugated with the pre-treated N3-tagged glycans via copper-free click reaction, achieving specific surface localization of UPDAs onto live cells.36 Upon 808 nm laser excitation, heat released from specifically localized UPDAs was monitored to comparatively study the heat shock response in cells, thus disclosing their molecular modes of cell death (Scheme 1).

Scheme 1. Specific localization of photothermal upconversion nanoparticles dissect distinguished cellular responses to heat stress.
**RESULTS AND DISCUSSION**

**Rational Designs and Characterizations of Upconversion Nanoheaters.** Figure 1A presented the design of our NIR light responsive nano-heaters, in which biocompatible polydopamines with high NIR spectral absorbance were coated on lanthanide doped upconversion nanoparticles (UPDA) to produce promising photothermal effect after 808 nm laser excitation. TEM images showed the homogeneous coating of polydopamine on UCNs with a thickness of 8 nm (Figure 1A). The hydrodynamic diameter in buffer solution was determined as 100.5 ± 19.13 nm through dynamic light scattering (DLS) analysis (Figure S1A). Meanwhile, upconversion luminescence characterized by blue emission peaked at 460 nm facilitates the applicable tracking of UPDA position in live cells (Figure S1B). We then modified UPDA with different chemical moieties, e.g. DBCO and PEI, on the particle surface (Figure S1C). The successful conjugation of DBCO on UPDA and effective cell chemistry with N\textsubscript{2} group was confirmed by the fluorescent conjugation through 5-carboxyfluorescein-azide (FAM-N\textsubscript{3}) with the optimal amount of DBCO of ~363 nmol/mg (Figure S2). Moreover, PEI was coated on UPDA for increasing the efficiency of cellular uptake, which further enhanced the subsequent localization in cytoplasm. There was no obvious morphology and shape difference observed among the modified nanoparticles (Figure S1D, E). Zeta potential measurement indicates a negative value of -10 mV for UPDA-DBCO, while a positive potential was found for UPDA-NH\textsubscript{2} (+16 mV) (Figure S1F), indicating its great possibility for cell uptake.

The obtained nano-heaters demonstrated the time and concentration dependent heat effects (Figure 1B, C) upon 808 nm light illumination, which was also observed even under deep tissue depth (Figure S19). Importantly, the UPDAs showed great stability in different pH condition (Figure S3, 4) and repeated cycles of photothermal treatment (Figure S5). Moreover, these particles indicated similarity in heating and natural cooling cycles in their photothermal performance (Figure 1D) as well as calculated to have comparable photothermal conversion efficiencies (Figure S6). These properties enable a reasonable comparison of heat response through different localization of UPDA in cells by introducing equal amount of nanoparticle in each position of interest.

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**Specific Cellular Localization of Nanoheaters.** For conjugation of nanoparticles on the cell membrane, the covalent glycan labelling approach was first conducted by introducing the N\textsubscript{2}-tagged glycans onto human lung adenocarcinoma epithelial (A549) cells. Upon feeding with Ac\textsubscript{2}ManNAz precursor (50 μM) for 48 hours, the A549 cells were incorporated with azido groups on the membranes through ubiquitous cellular metabolism (Figure S7). The N\textsubscript{2}-tagged cells were further incubated with UPDA-DBCO and dynamic imaging was performed, revealing the gradual localization of UPDA on cell membrane (Figure S8). The membrane accumulation of nanoparticles reached plateau after 1 h. Notably, extended incubation time (e.g. 2 h) resulted in slow cellular internalization, while majority of UPDA were still anchored on the cell membrane. Moreover, control studies by unmodified UPDA alone or untagged cells revealed negligible number of nanoparticles on the cell surface, which further consolidated the specificity of the labelling through the metabolic glycol-biosynthesis (Figure S9). During this process, care needs to be taken to maximize the quantity of UPDA on the cell surface with minimum internalization by optimizing the incubation time (e.g. 1 h) to make sure that the specific thermal response mainly originates from the membrane localized nano-heaters (Figure S8).
In addition, we examined the possibility for different localization of upconversion nanoheaters in live cells. Typically, the effective cellular distribution of nanoparticles inside cells can be obtained by incubation of A549 cells with UPDA-NH$_2$. The time-dependent cellular uptake was monitored by confocal and upconversion imaging analysis to optimize the comparable quantity of nanoparticles internalized as contrast to those UPDA-DBCO counterparts located on the membrane upon proper time incubation (e.g. 6h, Figure S10). Indeed, as shown in Figure 1E, different from the UPDA-DBCO labelled on the plasma membrane, the UPDA-NH$_2$ could be taken up efficiently and almost no membrane-located interference was observed in cell imaging. In order to monitor their contribution to cellular thermal response, we individually localized UPDA-DBCO and UPDA-NH$_2$ into different compartments, and then carried out ICP measurement to quantify the amount of incubated UPDA in live cells.$^{37,38}$ The relatively same number of nano-absorbers (e.g. a ratio of ~ 1 : 1.05) were precisely maintained on the cell membrane and cytoplasm, respectively (Figure S11), which promise a rational strategy for further comparative investigations.

**Heat Shock Proteins Expression upon Photothermal Stimulation.** Upon the localization of UPDA on the different positions in the cells, both the nanoheaters on membrane and cytoplasm were irradiated with 808 nm laser (1.3 W/cm$^2$) for thermolysis analysis. The heat effect was carefully monitored through the NIR thermograph to optimize the proper concentration (e.g. 200 µg/mL) and temperature increment (e.g. ~ 8.5 °C from 37 °C) (Figure 1C, S12) for effective photothermal response in live cells.$^{39}$ Prior to NIR light irradiation, there was no obvious thermolysis observed in the nanoparticle localized cells without laser illumination, suggesting negligible cell perturbation even under high concentration of nanoparticles (Figure S12A). After laser irradiation, cells with specific UPDA localization experienced significant thermolysis effect (Figure S12B), suggesting suitable concentration and light treatment for further photothermal study.

Notably, soon after the heat stress was exposed, the cells started to produce heat shock protein (HSP), as a cytoprotective response against the detrimental increase of temperature (Figure S13).$^{40}$ Three hours after the heat stress triggered by NIR laser irradiation, both groups of cells with different subcellular nano-heaters localization induced the significant production of HSP70, as indicated in green fluorescence from Alexa Fluor 488 labelled anti-Hsp70 antibody (Figure 2A, B). Controlled experiments showed the minimum expression of HSP70 in cells that lack UPDA treatment or without laser irradiation (Figure S14). Quantitative fluorescence-activated cell sorting (FACS) analysis demonstrated relatively equal amount of HSP70 expression at both 1 h and 3 h post-irradiation in each group of cells (Figure 2D, E, F). Although the detailed mechanism of such stimulated protection remains controversial, the heat induced native protein denaturation could be a possible driving force to stimulate the transcription of heat shock proteins upon binding to the promotor of the specific HSP gene.$^{41}$ Additionally, recent studies also indicated the possibility of membrane perturbation for cytoprotective HSP70 expression, in which cell surface disruption could compromise the membrane integrity and give rise in bulky membrane hyperfluidization.$^{42}$ To this end, we treated the live cells with benzyl alcohol (BA), one typical membrane perturbation reagent. Indeed, as shown in Figure 2C, the effective activation of HSP70 clearly suggested the possible involvement of membrane function and the integrity in cellular response to the stimulated stress. In our case, the accumulation of UPDA on membrane concentrated heat onto a confined area, which possesses low thermal conductivity, and may therefore contribute to larger temperature gradients and membrane disruption.$^{43,44}$ Apparently, UPDA membrane conjugated A549 cells indicated the well-organized surface structure with a specific residing of nanoparticles before NIR light exposure. However, 15 min post laser irradiation, the membrane tracker signal was disturbed, and the signal showed the releasing of cytoplasm components after 30 min (Figure S15). These results confirmed the occurrence of membrane perturbation during stimulation of UPDA localized on cell surface. Although the detailed processes involving HSPs expression require further investigation, the potential disruption of membrane integrity triggered by heat stimulation would be one noticeable perspective (Figure 2G).

**Figure 2.** Cellular analysis of photothermal effect and heat shock response. A) Confocal images of HSP70 expression in A549 cells treated with localized UPDA-NH$_2$. B) UPDA-DBCO, 3 h after photothermal stimulation, and C) Benzyl alcohol (50 mM). Scale bar in images: 40 µm. (λ$_{ex}$: 488 nm, λ$_{em}$: 515/30 nm). D, E) Flow cytometry analysis of HSP70 expression in different time points post-irradiation of cell with membrane and cytoplasm localized UPDA, respectively. Treated cells were stained with Alexa Fluor 488 labelled anti-Hsp70 antibody. F) Mean intensity of HSP70 levels. G) Potential mechanism of heat-shock protein expression.

**Distinguishable Photothermolysis at different subcellular locations.** We further examined the possible mode of cell death after NIR irradiation of nano-heaters located at different cellular compartments. Typically, cells with site-specific labelling of UPDA were irradiated and the standard AnnexinV/prodipidium iodide (AnnV/PI) co-staining were recruited to compare the cell death pathways. As shown in Figure 3A, heat from cytoplasm located UPDA stimulates outer membrane translocation of phosphatidylserine (PS) at early time duration after irradiation, as detected by green emission from FITC-AnnV binding protein. Upon further incubation, PI molecules were gradually
permeated into the nucleus. As contrast, cells with membrane conjugated UPDA experienced rapid intracellular uptake of PI molecule. Moreover, the level of apoptosis and necrosis were analysed by FACS. Figure 3B and S16 showed consistent results as observed from confocal imaging. Particularly, at 1 h and 3 h post irradiation, cells that internalized the UPDA encountered 4.96 % and 16.12 % of apoptosis at early stage, respectively. This group of cells faced secondary necrosis with approximately 31.15 % of total cells after 5 h incubation. Meanwhile, the membrane localized UPDA induced gradual increase in necrosis level from initial stage after illumination (4.48 % in 1 h to 47.09 % after 5 h) and minimum amount of early apoptosis was detected. Such different thermal response revealed that UPDA internalized cells experienced apoptotic process which remained membrane integrity at the primary stage after illumination. In contrast, membrane localized UPDA obviously caused membrane perturbation which allows prompt internalization of PI molecules, and therefore likely to proceed the cell death pathway to necrosis.

To further validate the distinguished cellular lethality, the molecular basis associated with light triggered thermolysis was investigated. First, we employed LDH leakage as an indicative assay for membrane integrity. As indicated in Figure 3C, irradiation of cell surface distributed UPDA could lead to a time dependent LDH release. Specifically, at 5 h post-irradiation, there were nearly 3 times increase in LDH level as compared to the initial amount observed in the same culture medium. Differently, LDH content in medium was found to rise up slightly in cytoplasm localized with UPDA and the rate of leakage was much slower than the former case of UPDA on the cell surface. These different outcomes further confirmed the presence of necrosis in cells with the membrane located by UPDA.

Secondly, we investigated the activation of caspase 3, a standard biomarker for apoptosis, after locally triggered heat stress by NIR light irradiation. The fluorescent signal from caspase 3 probes was analysed to monitor caspase 3 expression. As shown in Figure 3D, the level of caspase 3 enzyme in live cells with cytoplasm accumulation of nano-heaters uplifted around 2 times within 3 h post laser irradiation. Whereas, in the cells with particles conjugated on the plasma membrane, less enzyme expression was detected even prolonging the incubation time, up to 5 h post laser irradiation.

So far, apoptosis has well been recognized as one of standard pathways in the heat-stimulated cell dying, and lysosomal leakage of nano-heaters would be one critical step that initiates chain of events before activating caspase 3 in apoptosis (Figure 4A).45,46 Such initial step of cell dying was also observed in our cells with cytoplasmic UPDA localization under NIR light exposure. After being internalized, UPDAs were mostly loaded in the lysosome as confirmed by co-localization of lysosome tracker (green) and UPDA (red) with the yellow colour in the merged image (Figure S17). Nanoparticles were released from lysosomes presenting as a single red colour signal after 10 minutes NIR light illumination while negligible lysosomal escape was observed in the cell without laser irradiation (Figure 4B, S18). Principally, heat stress from nano-heaters disrupts the integrity of lysosomal membranes, resulting in lysosomal membrane permeability (LMP) that trigger cathepsin-mediated cell death pathway. At early stage of this process, a pro-apoptotic protein, Bid, can be cleaved to its truncated tBid and initialize the intrinsic mitochondria apoptosis pathway.47 Herein, we evaluated the level of tBid in two groups of cells with site specific localization of UPDA on membrane and in cytoplasm, respectively. Typically, immunofluorescence analysis exploiting tBid antibody and fluorescent secondary antibody were applied to label tBid protein. As indicated in Figure 4C, tBid was activated in cytoplasmic UPDA containing cells, revealed by the green fluorescence, while the membrane accumulated one showed lower intensity. Western blot experiment was also performed, and the results clearly indicated the decreased amount of the full-length Bid and the enhanced quantity of tBid in the UPDA internalized cells in response to photothermal stress. Meanwhile, the similar irradiation of membrane located UPDA cells resulted in an insignificant tBid expression in comparison with the control without laser treatment (Figure 4D). These distinctive observations demonstrated that the localization of UPDA in lysosome organelle and their subsequent liberation can convert the Bid protein to its activated form, tBid, which thus functions as a key factor to initialize apoptosis. These fundamental biological assays clearly indicated the importance of subcellular position of nano-stimulators for their differentiated heat response which will therefore affect further cell lethality significantly.
In summary, NIR light responsive upconversion nano-heaters positioned at different cellular location can differently affect the process of cell responses on the photothermal stress. On one hand, we employed the intrinsic glycan metabolic process to introduce azido group on cell surface which subsequently conjugated the DBCO modified UPDA onto the cell membrane. Upon 808 nm laser irradiation, temperature elevation caused by UPDA-DBCO promoted the destruction of the plasma membrane, thus stimulating the expression of HSP70 and UPDA-NH2 at 15 and 30 min after 10 minutes 808 nm irradiation (1.3 Wcm⁻²). White arrows indicate location of UPDA-NH2 after lysosomal release. Green: Lysosomal tracker, Alexa Fluor 488 (λex: 488 nm, λem: 515/30 nm), red: UPDA (λex: 543 nm, λem: 580/50 nm). C) Images of tBid expression in cells with site-specific nanoparticle localization at 5 h post-irradiation. Blue: YNanoFlour 488 λex: 405 nm, λem: 460/50 nm, green: Alexa Fluor 488 (λex: 488 nm, λem: 515/30 nm). Scale bar: 20 μm. D) Western-blot analysis of Bid activation after irradiating the site-specific located UPDA. GAPDH was used as internal control.

CONCLUSION

In summary, NIR light responsive upconversion nano-heaters positioned at different cellular location can differently affect the process of cell responses on the photothermal stress. On one hand, we employed the intrinsic glycan metabolic process to introduce azido group on cell surface which subsequently conjugated the DBCO modified UPDA onto the cell membrane. Upon 808 nm laser irradiation, temperature elevation caused by UPDA-DBCO promoted the destruction of the plasma membrane, thus stimulating the expression of HSP70 and directing the cell death to necrosis. Whereas, UPDA-NH2 were internalized by cells, where these cytoplasm localized nanoparticles not only induced HSP70 expression upon light-triggered temperature increment as those UPDAs on the cell surface, but more significantly, they can also promote lysosomal membrane permeability upon photothermal treatment, thereby inducing the activation of tBid cleavage and resulting in a differentiated cell death, apoptosis. Our study specifically revealed the molecular basis of the critical importance of photothermal nanoparticles with organelle-specific localization in manipulating cellular responses. With the ubiquitous properties of these organelles responsible for multiple cellular pathways, we expect that the integration with innovative nanotechnology designs could offer great opportunity to precisely regulate other cell functions and better understand the cellular basis of photothermal responses. Prospectively, this strategy could render optimized practical conditions for future personalized nanomedicine.

EXPERIMENTAL PROCEDURES

Materials. Y(CH₃CO₂)₃, Yb(CH₃CO₂)₃, Tm(CH₃CO₂)₃, Nd(CH₃CO₂)₃, oleic acid, 1-octadecene, NH₄F, NaOH, peracetylated N-azuidoacetylmannosamine (Ac₆ManNAz), in vitro toxicity assay kit (TOX8, resazurin based), Hoechst 33342 (bisBenzimideH 33342 trihydrochloride), Dibenzylecloytyamine (DBCO-NH2, DBCO-Cy3), 5-carboxyfluorescein-azide (FAM-N₃), dopamine hydrochloride, branched polyethyleneimine (PEI₃₀₀₀), and Annexin V PI detection kit, caspase-3 fluorometric assay kit were purchased from Sigma-Aldrich. CytoTox 96 nonradioactive cytotoxicity assay kit were purchased from Promega. Rabbit monoclonal [Y8] to Bid, Goat Anti-Rabbit IgG H&L (Alexa Fluor 488) were purchased from Abcam. Bid p15 Polyclonal Antibody was purchased from Thermal Fisher. Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium (MEM), fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA, Cell Mask™ deep red plasma membrane stain kit (CMSK-Cy5). All the commercially reagents were used as received unless otherwise noted. The human lung adenocarcinoma epithelial cell line (A549) were cultured in Dulbecco's modified eagle medium (DMEM) medium with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO₂.

Characterization and measurement. Transmission electron microscope (TEM) images were obtained using a FEI EM208S TEM (Philips) operated at 100 kV. Dynamic light scattering (DLS) and zeta potential measurements were performed by Brookhaven 90 Plus Nanoparticle Size Analyzer. Fluorescence emission spectra were recorded on a RF-5301PC Spectro fluorophotometer (Shimadzu, Japan) at room temperature. Photothermal performance was monitored by FLIR E60 thermal imaging camera. The cell viabilities were measured by a Tecan’s Infinite M200 microplate reader. Confocal imaging of cells was carried out on Carl Zeiss LSM 800 confocal laser microscope (Germany). Photo-irradiation experiments were performed with an 808 nm NIR diode laser (Changchun New Industries Optoelectronics Technology Co., Ltd., China). Flow cytometry analysis was performed using BD LSRFortessa™ X-20 flow cytometer.

Synthesis of PDA-coated UCNP (UPDA). The synthesis of NaNF₃-Yb/Tm/Nd (30/0.5/1%) @NaYF₅-Nd (20%) core-shell upconversion nanoparticles were synthesized based on the standard method published by our group previously. In a flask containing 5 ml of cyclohexane, 5 mg of the prepared core-shell UCNs were added together with 0.4 ml of Igepal CO-520 and sonicated for 30 min. Then, 40 μL (28 wt % in water) ammonium hydroxide was carefully added into the solution and stirred for 30 min. The aqueous solution of dopamine hydrochloride was prepared from 16 mg of dopamine hydrochloride in 100 μL of deionized water (for 8 nm polydopamine shell thickness) before slow addition of the above mixture under ultrasonication at a rate of 5 μLmin⁻¹. The reaction mixture was stirred for an additional 24 h. The UPDAs
were precipitated by adding ethanol, collected by centrifugation and washed several times with ethanol and water.

**Synthesis of UPDA-DBCO, UPDA-NH₂ and Rhodamine-UPDA.** The polydopamine coated UCNs were covalently modified with DBCO-NH₂ by typical Michael addition and/or Schiff-based reaction with catechol and amine groups on UPDA. Briefly, the as-prepared UPDA (5 mg) were dissolved in Tris buffer solution (pH 8.5) with a mixture of deionized water and ethanol (2:1) as solvent. DBCO-NH₂ (5 mg) was dissolved in ethanol before carefully added into the prepared mixture. After 24 h magnetic stirring at room temperature, the DBCO-UPDA was obtained by centrifugation (14,000 rpm, 10 min) and washed by ethanol three times to remove excess reagent. The precipitate was stored in fridge before use. The amount of DBCO moiety on the surface of UCNs was determined based on the fluorescence of conjugated 5-carboxyfluorescein-azide (FAM-N₃) molecules by copper-free click chemistry reaction. Generally, the as-prepared DBCO-UPDA (1 mg) were dissolved in DMSO (200 μL) containing FAM-N₃ (0.2 mg, 43.6 μmol) and stirred in the dark for 2 h. The nanoparticle was centrifuged (14,000 rpm, 10 min) and washed by ethanol three times to remove excess reagent. The precipitate was re-dispersed in 1 mL DMSO for later determination of the DBCO binding efficiency by measuring the fluorescence at 540 nm, excitation: 488 nm.

The synthesis of UPDA-NH₂ was conducted by dispersing the UPDA (5 mg) in a 1 mg/ml branched polyethyleneimine (PEI₂₀₀₀) solution, stirred for 12 h. The obtained UDPA-NH₂ was collected by centrifugation (14,000 rpm, 10 min) and washed with ethanol and water.

For UPDA to be tracked from confocal imaging, Rhodamine B-UPDAs were synthesized. Specifically, 1 mg of Rhodamine B was activated by EDC/NHS (1:1 molar ratio) in DMSO at room temperature for 2 h. The mixture was then added dropwise into the UPDA aqueous solution (5 mg UPDA) and stirred vigorously overnight. The obtained nanoparticles were collected by centrifuging at (14,000 rpm, 10 min) and washed with ethanol 3 times for removing unbound dyes.

**Photothermal performance measurement.** 0.2 mL aqueous dispersion of different concentration of UPDA nanocomposites were irradiated with an 808 nm laser at 1.3 W cm⁻² for 10 min and monitored the temperature changes by an infrared thermal camera (E60, FLIR).

**Cell membrane conjugation of DBCO-functionalized agents.** The human lung adenocarcinoma epithelial (A549) cell line were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 100 units mL⁻¹ penicillin and 100 μg mL⁻¹ streptomycin and maintained in a humidified incubator with 5% CO₂ at 37 °C. The cells were subsequently incubated with a density of 5 × 10⁶ cells in an ibidi dish (35 mm, plastic bottom) in 1 mL DMEM medium with Ac₂ManNAz (50 μM) for 48 hours. The resulted azido-labelled cell membrane was then stained with DBCO-Cy3 (10 μM, 30 min), CMSK-Cy5 (5 μM, 10 min) and Hoechst 33342 (1 μM, 30 min) separately. The cells without azido sugar treatment and azido-labelled cell membrane blocked with DBCO-NH₂ (50 μM, 30 min) were used as control experiments.

**Conjugation of UPDA-DBCO on cell membrane.** 5 × 10⁵ A549 cells were cultured in an ibidi dish (35 mm, plastic bottom) and incubated with Ac₂ManNAz (50 μM) in DMEM medium at 37 °C, 5% CO₂ for 48 h. The cells were then washed with PBS (pH 7.4) three times before adding 200 μg/ml of UPDA-DBCO in 1 mL DMEM medium. After further 1-hour incubation, the cells were washed carefully with PBS three times and the specific localization of nanoparticles was monitored by a confocal microscope with CMSK-Cy5 as a membrane tracker.

**UPDA-NH₂ localization in the cytoplasm.** 5 × 10⁵ A549 cells were cultured in an ibidi dish (35 mm, plastic bottom) in DMEM medium. After 48 h, the cells were washed with PBS three times before adding 200 μg/ml UPDA-NH₂ and further incubated for 6 h. The specific distribution of nanoparticles inside the cells was monitored using a confocal microscope with CMSK-Cy5 as a membrane tracker.

**ICP quantification of UPDA.** Two groups of cells after specific localization of UPDA (using optimized condition for each localization) was washed three time with PBS. After that, the cells were collected by 0.25% trypsin-EDTA, centrifuged at high-speed (10, 000 rpm, 10 min) and treated with 70% nitric acid for 24 h. The clear solution was diluted in water (2% nitric acid), filtered with 0.22 μm filter and analyzed for yttrium (Y³⁺) concentration by ICP-OES.

**Cell viability test.** The A549 cells were seeded with a density of 1 × 10⁴ cells per well in 200 μL DMEM in the 96-wells plate and incubated with 50 μM of Ac₂ManNAz for another 48 h before adding UPDA-DBCO with different concentration in 1h for membrane conjugation and incubated for 24h. Meanwhile, other A549 cells were seeded with a density of 1 × 10⁴ cells per well in 200 μL DMEM in the 96-wells plate and incubated for 48 h before washed carefully with PBS and incubated with different concentration of UPDA-NH₂ in culture medium for internalization during 24 h. After that, the cell treated with different nanoparticles were washed with PBS and fresh cell culture medium containing TOX8 was added to the wells, and the plate was incubated for another 3 h. The fluorescence at 590 nm was measured by a Tecan’s Infinite M200 microplate reader with 560 nm excitation. Cell viability was expressed by the ratio of the fluorescence of cells conjugated with nanoparticles to that of control.

For photothermal cell viability test, A549 cells with specific conjugation of UPDA was monitored with previous mentioned incubation time to have equal number of nanoparticles in each group of cells before being irradiated with 1.3 W cm⁻² 808 nm laser for 10 minutes. The photothermal treated cells was then further incubated for 24 h before being washed with PBS and measured the viability using the same TOX8 based method described above.

**Hsp70-Expression Analysis.** UPDA membrane conjugated and UPDA cytoplasm localization of A549 cells were seeded on the 8-well ibidi dishes containing 5 × 10⁴ cells per well in 200 μL DMEM media. After 10 min irradiation with 1.3 W cm⁻² 808 nm laser irradiation, the media was removed, and cells were washed with PBS 3 times. After additional incubation for 5 min, 1 h, 3 h, 5 h, A549 cells were washed and fixed with 4% paraformaldehyde solution for 15 min at room temperature. After that A549 cells were washed with PBS before staining with anti-Hsp70 antibody conjugated with Alexa Fluor 488 (Biolegend Alexa Fluor 488 anti-Hsp70) at room temperature for 15 min. Stained A549 cells were washed with PBS and observed with confocal microscope.

For flow cytometry (FCM) analysis, A549 cells were seeded in 6 well dishes in 2 mL DMEM cell culture media with density of 1 × 10⁴ cells per well and allowed for site-specific localization of UPDA-DBCO and UPDA-NH₂ (200 μg/ml for each). After photothermal treatment as above-mentioned method, cells were further incubated for 1 h or 3 h before collected by 0.25%
trypsin-EDTA, fixed with 4% paraformaldehyde solution for 15 min at room temperature. The collected cells of each group were washed with PBS before staining with anti-Hsp70 antibody conjugated with Alexa Fluor 488 (Biolegend Alexa Fluor 488 anti-Hsp70) at room temperature for 15 min. Stained A549 cells were washed with PBS and analysed with BD LSRFortessa™ X-20 flow cytometer.

For benzyl alcohol induced HSP70 expression, 1×10^6 in ibidi dished (35 mm) was incubated with 50 mM of BA in cell culture DMEM media for 1 hour. After that, the cells were carefully washed with PBS and fixed with 4% paraformaldehyde solution for 15 min at room temperature before staining with anti-Hsp70 antibody conjugated with Alexa Fluor 488 (Biolegend Alexa Fluor 488 anti-Hsp70) at room temperature for 15 min. Stained A549 cells were washed with PBS and observed with confocal microscope, using 488 nm laser and 480 ± 25 nm filter. For FCM analysis, cells with the same BA treatment condition were collected by 0.25% trypsin-EDTA, fixed with 4% paraformaldehyde solution for 15 min at room temperature. The collected cells were then washed and stained with anti-Hsp70 antibody before analysed with BD LSRFortessa™ X-20 flow cytometer.

**Apoptosis/ necrosis imaging and FCM analysis.** Double-stain molecular probe consisting of Annexin V-Alexa Fluor 488 (AnnV) for detecting apoptosis cells and propidium iodide (PI) staining necrotic cells was used to investigate the membrane integrity as well as cell necrosis and apoptosis. Typically, 5 µL volume of AnnV (100 µg/mL) and a 1 µL volume of PI (100 µg/mL) were added to each group of cells with different site-specific UPDA localization after photothermal treatment in 100 µL of 1× annexin-binding buffer. After staining, fluorescence images were taken using a Carl Zeiss LSM 800 confocal laser microscope and the quantity of early apoptosis, late apoptosis and necrosis cells was analysed by BD LSRFortessa™ X-20 flow cytometer.

**LDH release assay.** For investigation of necrosis, Cyto Tox 96 assay (Promega) was carried out according to the manufacturer’s instructions. Generally, each group of site-specific UPDA localization cells was grown in 96 well-plate with 1×10^4 cells/well density. Cells was washed with PBS before photothermal treatment. After 1, 3, and 5 hours additional incubation, the sample solution was incubated with LDH substrate buffer solution in dark for 30 minutes. Then, stop solution was added before measuring the absorbance at 490 nm a Tecxan’s Infinite M200 microplate reader. Each experiment was repeated three times and the results was reported in average values and standard deviations.

**Caspase 3 activity assay.** Caspase 3 activity assay (Caspase 3 Assay Kit, Fluorometric, Sigma-Aldrich) was carried out according to the manufacturer’s instructions. Typically, 1×10^4 site-specific UPDA localization cells were grown in each well of 96 well plate. After photothermal treatment, cells were washed with PBS and lysis buffer was added. The cells were then allowed to be lysed in 0 °C for 20 min. Consequently, assay buffer containing caspase 3 probe was added and further incubated for 30 min. The increase in fluorescence at 460 nm under 360 nm excitation of each sample solution was recorded by a Varian Cary Eclipse Fluorescence Spectrophotometer. Each experiment was repeated three times and the results was reported in average values and standard deviations.

**Immunofluorescence analysis of tBid protein.** Each group of site-specific localization of UPDA cell with density of 5×10^4 cells in each well of 8-well ibidi-dishes was experienced photothermal treatment as described above. After 5 h, the cells were fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.25% Triton™ X-100 for 10 minutes and blocked with 5% BSA for 1 hour at room temperature. The cells were then labelled with Bid (p15) Rabbit polyclonal IgG secondary antibody was stained for 30 minutes at room temperature. Nucleus was stained with Hoechst 33342. The induction of Bid cleavage site (p15 tBID) was visualized with Carl Zeiss LSM 800 microscope. (Hoechst 33342: Ex = 405 nm, Em = 460/50 nm; Alexa Fluor 488: Ex = 488 nm, Em = 515/30 nm).

**Western-blot analysis of Bid protein activation.** The lysates of each group of site-specific localization UPDA at 5 h after photothermal treatment were collected and centrifuged at 12,000 × g for 10 min at 4 °C. Protein levels in supernatants were determined using Nanodrop and equalized to the same concentration and boiled for 10 min with SDS-PAGE sample loading buffer before being separated using SDS-PAGE and transferred to the PVDF membrane. The membrane was then blocked with 5% BSA-TBST blocking buffer overnight at 4 °C. Subsequently, full length BID primary antibody (1:2000) (Rabbit monoclonal [Y8] to Bid, abcam) and p15 Bid cleavage site-specific antibody (1:1000) were incubated at room temperature for 2 hours in 2% BSA-TBST buffer. After series of washing, the goat-anti rabbit IGG (H&L) secondary antibody was added and incubated for 1 hour in 2% BSA-TBST. All signals were developed using Super Signal West Femto kit, visualized using myECL imager.

**ASSOCIATED CONTENT**

The Supporting Information file contains further characterization of nanoparticles (TEM, DLS, stability tests, photothermal conversion efficiency); optimized treatment conditions of nanoparticles to achieve specific cell compartment localizations; further details and control experiments of heat shock responses and cell death mechanism. This material is available free of charge via the Internet at http://pubs.acs.org

The authors declare no competing financial interest.

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The manuscript was written through contributions of all authors.

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